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Microsatellite DNA Variation in Sandbar Sharks (*Carcharhinus plumbeus*) from the Gulf of Mexico and Mid-Atlantic Bight

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Three polymorphic DNA microsatellite loci were developed in the sandbar shark (*Carcharhinus plumbeus*) and used to test the hypothesis that sharks from the mid-Atlantic Bight and western Gulf of Mexico comprise a single genetic stock. No significant differences in microsatellite allele frequencies were detected, a finding consistent with the null hypothesis. Polymorphic microsatellite loci appear to be scarce in sandbar sharks relative to other fish species. The three loci examined in this study had relatively low levels of heterozygosity, most likely attributable to the small number of dinucleotide repeat units per locus.

THE sandbar shark (*Carcharhinus plumbeus*) is a common coastal species distributed throughout tropical and warm-temperate oceans (Compagno, 1984). The commercial value of its large fins and high-quality meat support directed fisheries throughout the world. Sound conservation and management of any exploited fishery requires a fundamental understanding of population structure of the species involved. Genetic characters often have proven useful in discerning population structure, even in species with the potential for long-distance (genetic) dispersal (Palumbi, 1994; Gold and Richardson, 1998).

Heist et al. (1995) found no significant differences in frequencies of either allozyme alleles or mitochondrial (mt) DNA haplotypes between samples of sandbar sharks from the Gulf of Mexico (Gulf) and Mid-Atlantic Bight (western North Atlantic). However, their study was constrained by low levels of intrapopulational variation in both genetic markers. In this study, we assayed many of the same individuals studied by Heist et al. (1995) for variation in three nuclear-encoded microsatellite loci. Microsatellite loci are short repeat motifs that are less than six base-pairs (bp) in length (Weber, 1990). Because microsatellite loci typically exhibit much greater variability than allozymes or mtDNA (Bentzen et al., 1996; Tessier et al., 1997), they would appear to be especially useful for species (e.g., sandbar shark) in which allozyme and/or mtDNA variation is low. In addition, because a large number of microsatellite loci generally exist in vertebrate genomes (Tautz et al., 1986; Colburne et al., 1996), microsatellites provide the opportunity to assay multiple loci that can be used to provide independent measures of gene flow. Our intent was to test further whether sandbar sharks in the Gulf and western North Atlantic belong to the same subpopulation (stock). To our knowledge, this is the first published report on microsatellite loci in an elasmobranch.

MATERIALS AND METHODS

Sandbar sharks from the mid-Atlantic Bight (n = 45) were collected using longlines on a cruise in the mouth of Chesapeake Bay in 1992. Samples from the Gulf of Mexico (n = 26) included individuals collected from waters off Mexico (n = 5) and Texas (n = 21) between 1993 and 1996. Samples collected prior to 1995 were stored at −70 C. Samples collected in 1995 and 1996 were stored at room temperature in 10X Longmire's buffer (0.1 M Tris, 0.1 M NaCl, 0.1 M EDTA, 0.5% SDS, pH 8.0).

For isolation and characterization of individual microsatellites from sandbar sharks, genomic DNA was isolated from frozen heart tissue as in Gold and Richardson (1991). DNAs were digested with restriction enzyme *Sau*3AI and electrophoresed against a 100-base pair (bp) size standard on 1.4% TAE agarose gels. Fragments spanning 400–1000 bp were excised from the gel with a scalpel and extracted from the gel matrix via electroelution. Fragments were concentrated by ethanol precipitation and ligated into a polycloning site within the ampicillin resistance gene of a Pgem −3Zf(+) vector that had been digested with *Bam*HI and dephosphorylated. Ligation was carried out using T4 DNA ligase at 16 C for 12–16 h. Bacterial (*E. coli* Electromax DH10B) cells were transformed and plated on to LB agar plates with 50ng/μl ampicillin. Plates were spread with IPTG and X-gal to facilitate selection of colonies with intact inserts via blue/white colony selection.

Positive (white) colonies were picked with sterile toothpicks and grown in 96-well microtiter plates containing 140 μl LB plus ampicillin (50ng/μl). Following overnight incubation at 37 C, the volume of wells was brought to 200 μl

TABLE 1. PCR PRIMERS DEVELOPED IN SANDBAR SHARKS (*Carcharhinus plumbeus*) AND ANNEALING TEMPERATURES USED TO AMPLIFY PRODUCTS.

Locus	Annealing temperature	Primer orientation	Primer sequence
*Cpl*6	60 C	Forward	5′ ACCAGCAGGCATCTCAAACA 3′
		Reverse	5′ CTTTAGCTCCCGCATCAGAG 3′
*Cpl*38	56 C	Forward	5′ TCCAAGCTGGGAATTGAAC 3′
		Reverse	5′ CTGCCGAAAGAGTTGAAGG 3′
*Cpl*39	56 C	Forward	5′ TACCTGCCACAAAACCTGAC 3′
		Reverse	5′ GCCTTTACAGATGCCAGTGA 3′

with addition of 60 µl 50% glycerol in water. Solutions were stored at −70 C until screening. A total of 2880 colonies was screened with the aid of a Beckman Biomek 2000 workstation. A stainless steel 96-pin replicator tool was used to simultaneously collect cells from each well of a microtiter plate and inoculate a nylon membrane (hybond) on top of a slab of the LB agar plus ampicillin. Each colony was spotted twice to screen for false positives. Following overnight incubation at 37 C, colonies were lysed, and DNA was fixed to the membrane following Sambrook et al. (1989). Radiolabeled hybridization probe was prepared by labeling a mixture of repetetive oligonucleotides [$(CA)_{15}$, $(GA)_{15}$, $(ATT)_7$, $(CCT)_7$, $(GACA)_8$]. Equimolar amounts of each oligonucleotide were combined into a single T4 polynucleotide kinase reaction that exchanged the terminal phosphate group with γ-^{32}P. Membranes were prehybridized for two hours and then hybridized with a radiolabeled probe overnight at 48 C. Dried membranes were autoradiographed for 36 h with intensifying screens. Positive colonies were retrieved from the library and grown in LB-AMP medium at 37 C overnight with gentle shaking.

Positive clones were sequenced from both ends using standard M13 sequencing primers on an IBI 373 or 377 automated sequencer. Primers for PCR within the region flanking the repeat motifs were selected using the Oligo 4.0 software package (W. Rychlik, Oligo 4.0 Macintosh, National Biosciences, Inc., Plymouth, MN, 1992, unpubl.), and PCR was performed under a variety of conditions to optimize production of high yields of target sequence and minimize additional fragments. Once appropriate PCR conditions were obtained, microsatellite loci were amplified from genomic DNA of 72 individual sharks. Alleles at individual shark loci were scored on denaturing polyacrylamide gels. A known DNA sequence was used as a size standard. Genotypic frequencies at each microsatellite locus were analyzed for deviations from Hardy-Weinberg equilibrium by pooling rare genotypes and applying the EXACTP option of the HDYWBG step in the BIOSYS-1.7 package of Swofford and Selander (1981). Homogeneity of allele frequencies was tested using the randomization procedure of Roff and Bentzen (1989).

Two measures of population structure were estimated: Weir and Cockerham's Θ (Weir and Cockerham, 1984), an unbiased estimator of Wright's F_{ST} (Wright, 1969), computed using Arlequin 1.1 (S. Schneider, J.-M. Kueffer, D. Roessli, and L. Excoffier, Arlequin: a software for population genetic data analysis, vers. 1.1, Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva, 1997, unpubl.) as described in Michalakis and Excoffier (1996); and Slatkin's R_{ST} (Slatkin, 1995), estimated using RST CALC (Goodman, 1997). R_{ST} considers the squared size differences between alleles and is more appropriate for loci that evolve via stepwise mutations. Statistical significance of Θ and R_{ST} was assessed by random permutations (1000 trials per comparison) within the Arelquin 1.1 and RST CALC.

RESULTS AND DISCUSSION

Of 2880 clones of sandbar shark DNA screened for microsatellites by probe-hybridization reactions, 21 were positive for microsatellite repeat motifs. Eighteen clones were fully sequenced. All but one of the clones sequenced contained identifiable repeat motifs; three clones were duplicates. Of 14 clones that contained unique repeat motifs, 10 were $(CA)_n$, three were $(GA)_n$, and one was $(GACA)_3$. Because many of the repeat motifs were either very short (fewer than five repeat units), close to the end of the cloned sequence (thus precluding design of a suitable primer), or had very low levels of polymorphism, only three sets of PCR primers were used to score all individuals (Table 1). Sequences of the clones, includ-

TABLE 2. OBSERVED GENOTYPES (LENGTHS OF ALLELES IN BASE PAIRS) AND EXPECTED HETEROZYGOSITIES OF MICROSATELLITE LOCI IN SANDBAR SHARKS (*Carcharhinus plumbeus*).

Locus	Genotype	Atlantic	Gulf	H_{exp}
Cpl-6	153/151	12	5	0.226
	151/151	33	21	
Cpl-38	**301/301**	**42**	**25**	**0.067**
	301/299	3	1	
Cpl-39	283/283	2	0	0.535
	283/281	8	3	
	283/277	1	0	
	281/281	33	22	
	281/277	1	1	

TABLE 3. PROBABILITY OF HOMOGENEITY OF ALLELE FREQUENCY, WEIR AND COCKERHAM'S Θ, AND SLATKIN'S R_{ST} OF POLYMORPHIC MICROSATELLITE LOCI IN SANDBAR SHARKS (*Carcharhinus plumbeus*).

Locus	P	Θ	R_{ST}
Cpl-6	0.394	−0.010	−0.008
Cpl-38	0.350	−0.006	−0.010
Cpl-39	0.713	0.018	0.008
Total	—	0.002	−0.003

ing repeat motifs, are available through GenBank (Accession Numbers AF067410, AF067411, and AF067412). A fourth locus produced a single observed heterozygote in 66 individuals screened (locus Cpl-34, GenBank Accession Number AF069503).

Each polymorphic microsatellite locus possessed either two or three alleles; expected heterozygosities ranged from 0.068–0.535 (Table 2). Number of alleles and heterozygosity values observed in sandbar sharks are far fewer and lower, respectively, than those observed in other fishes studied to date. Examples include bluegill sunfish (Colburne et al., 1996); cod, rainbow trout, and Atlantic salmon (Brooker et al., 1994); and bluefin tuna (Broughton and Gold, 1997). However, heterozygosity observed here for the three microsatellite loci was higher than that observed by Heist et al. (1995) for allozyme loci where mean heterozygosity was 0.005. No significant deviations from Hardy-Weinberg equilibrium were detected, either within samples or in combined samples for any locus. No significant differences in allele frequency were detected between sandbar sharks from the mid-Atlantic Bight and Gulf of Mexico. Tests of allele frequency homogeneity at each locus were nonsignificant (Table 3), as were values of Θ ($P = 0.324$) and R_{ST} ($P = 0.277$).

Results of this study, the previous genetic study of Heist et al. (1995), and tagging data (Casey and Kohler, 1991) corroborate the hypothesis that sandbar sharks in the mid-Atlantic Bight and Gulf of Mexico waters comprise a single genetic subpopulation or stock. Although Springer (1960) suggested that there are two separate breeding areas for sandbar sharks in the western North Atlantic, results of this study and those of Heist et al. (1995) record no significant differences in allele frequencies for sharks collected from locations as distant as Virginia and Veracruz, Mexico. Furthermore, sandbar sharks tagged in the waters of New York have been recovered in Mexican waters (Casey and Kohler, 1991).

Based on the small number of positive clones detected in our screening of the sandbar shark genomic library as compared to results in other fish species (Colburne et al., 1996; Brooker et al., 1994), it may be that microsatellite loci are rare in sharks. We are currently carrying out experiments to examine this issue further. Techniques for producing "enriched" microsatellite libraries where DNA fragments that contain repeat motifs are selected prior to cloning have been described recently (Kijas et al., 1994; Walbieser, 1995) and may prove useful on species with low microsatellite diversity.

The low microsatellite heterozygosity in sandbar sharks is concordant with levels of allozyme heterozygosity and mitochondrial DNA variation in a previous study (Heist et al., 1995). Sandbar sharks are a long-lived species; individuals do not mature until approximately 15 years of age (Sminkey and Musick, 1995). Low levels of genetic variation in long-lived organisms has been noted previously and has been hypothesized to be due to perhaps the slow pace of germ-line DNA replication or to low metabolic rates (Avise et al., 1992; Martin and Palumbi, 1993). There is evidence that the "molecular clock" for mitochondrial DNA in sharks is slow relative to other vertebrates (Martin et al., 1992). However, the low level of microsatellite variability observed in our study may be due to the small number of uninterrupted repeats in the loci assayed. The cloned alleles of loci *Cpl*-6, *Cpl*-38, and *Cpl*-39 possessed relatively low numbers of perfect repeat units (eight, six, and nine, respectively), and Weber (1990) observed that human microsatellites typically are not highly polymorphic unless they contain at least 10 uninterrupted repeat units. Moreover, Shug et al. (1997) found that microsatellites with low numbers of perfect repeat units in *Drosophila*

had low mutation rates. If allelic variation is entirely due to changes in the number of repeats in the longest uninterrupted motif, then the largest number of repeats observed in any single individual of sandbar sharks was eight, six, and 10, respectively, for loci *Cpl*-6, *Cpl*-38, and *Cpl*-39. Heterozygosities at these three loci rank concordantly with mean and maximum numbers of repeats (i.e., *Cpl*-39 > *Cpl*-6 > *Cpl*-38). In comparison, a microsatellite developed in blacktip shark (*Carcharhinus limbatus*) has a perfect repeat length of 19 repeats in the cloned sequence, possesses at least 20 alleles, and has an expected heterozygosity of 0.88 in a sample of 131 individuals (unpubl.). Because the blacktip shark has a very similar life history to the sandbar shark, we believe the low heterozygosity in sandbar shark microsatellites is a characteristic of these particular loci and is not representative of carhcarhinid sharks. However, microsatellites were similarly rare in blacktip sharks, and most loci had few repeats, suggesting that polymorphic microsatellites in carcharhinid sharks occur at a low frequency relative to teleosts and mammals.

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